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BDRL D/A ltr, 24 Nov 1971

AD 291 419

HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON

Eighth Quarterly Report of Progress

on

Research Project Number 4B04-14-004
Order Number FDO-5013

April 1 - June 30, 1962

Conducted by

Milk and Food Research, SEC

for the

U. S. Army Biological Laboratories
Fort Detrick, Maryland

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
Robert A. Taft Sanitary Engineering Center
Cincinnati, Ohio
August 1962

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ABSTRACT

The observations previously reported relating to the antigenic characteristics of an acid coupled conjugate of paralytic shellfish poison and formalinized bovine serum albumin (PSP-HCHO-BSA) have been confirmed. This antigen elicits weak antibody responses in rabbits which may be demonstrated by precipitin reactions between serum and homologous antigen and by the protective capacity of the serum against the lethal effects of PSP in white mice.

Additional studies on the chemical state of PSP in *G. catenella* and toxic clam siphons have failed to uncover any evidence to indicate that the PSP exists as a stable protein conjugate. The apparent differences in toxicity of PSP isolated by the several techniques employed can be accounted for largely by the influence of pH on the toxicity of PSP.

CONTENTS

HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON

I. Introduction

In a previous quarterly report (1) evidence was presented to indicate that conjugates of paralytic shellfish poison and formalin treated bovine serum albumin (PSP-HCHO-BSA) elicited production of antibodies in rabbits. The serum from rabbits which was capable of precipitating the homologous antigen was found also to be capable of protecting white mice against the lethal effects of paralytic shellfish poison (PSP). The experimental portion of this report includes a summary of the studies confirming and extending these observations, some additional information on the nature of PSP in toxic clams and Gonyaulax catenella, and a report on the isolation and purification of PSP from toxic clam siphons.

II. Experimental

Immunological investigation with PSP-HCHO-BSA.

In view of the successful production of rabbit antiserum capable of neutralizing the toxic effects of PSP as reported last quarter, additional studies were conducted to determine the reproducibility of these results.

Six 5-lb. rabbits were started on an immunization program employing an antigen preparation and injection schedule identical to that previously described in the Seventh Quarterly Report (1). Three of these rabbits received a total of 20 injections of antigen (equivalent to 50 mg. of BSA protein). Trial bleedings and titrations for precipitating and neutralizing antibodies were made after the animals received 25-and 50-mg. doses.

The three remaining rabbits died during the course of immunization as follows: rabbit 20 received 25 mg. (10 injections) and died of unknown causes during the 7-day interval between the 10th injection and trial bleeding-serum was not available; rabbit 22 received 22.5 mg. (9 injections), died within 10 minutes after 9th injection and serum was not collected; rabbit 23 received 30 mg. (12 injections), was found dead 2 days later at the time the 13th injection was to be administered, and serum was not available.

In order to facilitate a more conventional immunization schedule and reduce the man-hours associated with preparing fresh antigen prior to each injection, the antigen was lyophilized, stored at -10° C., reconstituted to volume, and injected at the 2.5 mg. (1.0 ml.) antigen protein level. This preparation proved to be quite toxic to rabbits when administered intravenously or when injected subcutaneously with Freund's adjuvant.

The results of titrations of sera collected from the rabbits which received a total of 25 mg. and 50 mg. of antigen protein are shown in Tables I and II respectively. These data reveal that generally poor antibody response as measured by precipitin reaction was elicited by the antigen. This finding is comparable to that reported last quarter. One rabbit out of the three displayed good titer after 25 mg. was administered but displayed little, if any, increase after administration of an additional 25 mg.

Tests were also performed with these sera to determine whether antibody capable of neutralizing PSP was formed. One ml. of undiluted serum number 19 (after 25 mg. immunization) was found to protect 19- to 21-gram mice (Hamilton Laboratories) against 0.3 micrograms PSP per ml. when administered I.P. Serum obtained from rabbit 19 prior to immunization did not provide any protection to the mice (See Table III). Serum from rabbit 19 collected after a total of 50 mg. antigen protein had been injected also protected mice when administered as a 1.0 ml. I.P. injection. Dilutions of this same serum (1:2, 1:3, and 1:5) did not prevent death of mice, but extended death times were observed in relation to the dilution used (See Table IV). Sera from rabbits 18 and 21 (See Table V) did not protect mice, and death times slightly in excess of those noted for the water controls were observed.

These data, together with those reported in the Seventh Quarterly Report (1) indicate PSP-HCHO-BSA antigen is capable of eliciting some neutralising antibody for toxin. However, it appears to be a weak antigen and dissociates readily with the release of sufficient toxin to kill the test animals unless extreme caution is exercised in handling the antigen. Because of these difficulties, little serum has been collected to date, and additional studies on alternate tests for demonstrating antibody to PSP have not been accomplished. At this point, it is difficult to assess the merits of this antigen. The inconsistent development of antibodies among test rabbits is observed too often to be ignored. On the other hand, the development of protective antibodies by a few rabbits

dicates that this type of antigen preparation be pursued further. Accordingly, six additional rabbits are presently receiving injections of freshly prepared antigen. It is anticipated that sufficient sera will be obtained from these rabbits to permit additional immunological investigations.

Attempts were made to immunize rabbits with aqueous extracts of Convaulax catenella cells as follows: 1.0 g. of wet, packed cells was homogenized in a Kontes K-88545, Size C (Duall) tissue grinder to yield approximately 99% cellular disruption. The homogenate was diluted 1:20 in distilled water, centrifuged at 10,000 g. for 10 minutes in the cold, and the supernate filtered through an ultra-fine sintered glass filter. This extract was assayed for PSP concentration by means of the mouse test and found to contain approximately 10 micrograms of PSP per ml. This indicated the original packed cells contained at least 200 micrograms of PSP per gram. Several 5- to 8-lb rabbits were injected with this material via various routes and at various volume levels, and it was found that initial 1.0 ml. I.V. or subcutaneous injections were lethal. Intraperitoneal injections up to 1.5-ml. volumes were tolerated. Consequently, three rabbits were immunized with this antigen by administering 1.5 ml. I.P. injections on an every-other-day schedule. Rabbit number 12 received a total of 16 injections (equivalent to 240 micrograms PSP) and trial bleedings and titrations were made after 4, 8, 12, and 16 injections. During the course of immunization, severe fibromas developed at the sites of injection in the muscle wall covering the peritoneal cavity. The animal has not been sacrificed to date, and it is difficult to ascertain the exact location

of these masses by external examination. Two additional rabbits (numbers 16 and 20) received respectively 60 micrograms and 45 micrograms equivalence of PSP by means of a similar injection schedule. Rabbit number 16 developed severe, mucoid diarrhea after 3 injections; whereas, rabbit number 20 developed the same syndrome after 4 injections. Injections were discontinued on these rabbits in an effort to alleviate their distress. Both rabbits were bled 7 days following the last injection (15 ml. by cardiac puncture). Rabbit 16 died three days after bleeding and rabbit 20 one day after bleeding. At the time of death, both rabbits were suffering from diarrhea, and both had developed the fibromas described above.

The sera obtained from these rabbits were titrated for precipitin titer against the homologous antigen and PSP-HCHO-BSA. These results are shown in Table VI and indicate that the material is not very antigenic and antibodies produced to it do not react with the conjugated PSP-HCHO-BSA antigen. These sera were also used to protect mice in neutralization tests with no success (See Table VII).

Preparation of antigen.

All PSP-HCHO-BSA antigen used in the immunological studies thus far, including those presented herein, has been prepared according to the procedure outlined in the Seventh Quarterly Report (1), unless otherwise specified.

Since preparation of small quantitites on a bi-weekly basis is inconvenient and time consuming, the feasibility of preparing large quantitites

of lyophilized antigen is presently under investigation. It is believed that storing the antigen preparation in a dry form may inhibit the previously observed dissociation of conjugated PSP from its protein carrier. Thus far, some success has been attained. It has been possible to lyophilize a dialyzed PSP-HCHO-BSA preparation and redissolve the dried powder. Initial intravenous injections into rabbits of these redissolved antigens have often resulted in death. However, the mouse bioassay of the reconstituted antigen indicates a toxicity equivalent to 2 μg/ml. of PSP. Although this amount of poison would not be expected to be fatal to rabbits, there may be further rapid dissociation of poison following injection. Also, when a dried antigen is redissolved, its initial toxicity doubles in less than one hour when held at 25°C., indicating very rapid dissociation of PSP under these conditions. In addition, the redissolved antigen shows a marked tendency to denature slowly with time and rapidly with moderate agitation. The toxic effects of this denaturation have not yet been determined. It is evident that additional work will be required to determine the relative toxicities, protein stabilities and rates of PSP dissociation in lyophilized and non-lyophilized antigen preparations.

Studies on the chemical state of PSP in toxic clam siphons.

In the last report (1), a summary of studies on the dialyzability of PSP contained in G. catenella was presented. These results indicated that PSP does not exist as a stable protein conjugate in these cells as received in the frozen state. Similar studies on the chemical state of the poison

in toxic clam siphons have now been undertaken. These studies revealed that when ground frozen clam siphons were dialyzed 24 hrs. at 25°C., there was quantitative recovery of PSP in the dialysates, indicating that, as in the case of frozen G. catenella cells, PSP does not exist in a stable protein conjugated form in toxic clam siphons.

While the dialysis studies described above were of considerable value in obtaining evidence for the presence or absence of PSP-protein conjugates in G. catenella cells and toxic clam siphons, any such conjugates actually present could be expected to be labile to hydrolysis. Hence, the possibility remained that the lengthy and rigorous dialysis procedures employed could have resulted in hydrolysis of such conjugates resulting in the release of free dialysable shellfish poison. In an effort to determine whether such did occur in the case of toxic clam siphons, additional more direct and rapid experimental methods were employed. The most extensive of these studies were on the toxic properties of saline extracts obtained from saline suspensions of homogenised clam siphons. These extracts were obtained by suspending 2-3 g. of homogenised clam siphon tissue in physiological saline to a total volume of 25 ml., centrifuging at 7500-8000 rpm for twenty minutes and decanting the somewhat opalescent supernatant. This procedure was carried out at 0-5°C. and 25-30°C. and in both cases the PSP was evenly distributed between the solid and liquid phases. However, as shown in Table VIII, bioassay of these extracts without acidification resulted in an apparent toxicity of only 73-78% of that potentially available upon acid hydrolysis of the same extracts.

This effect could be due either to the presence of PSP conjugated proteins having reduced toxicity or to the pH effect on the bioassay. In order to determine whether the soluble proteins contained conjugated PSP, they were precipitated under non-acidic conditions, acid hydrolyzed and bioassayed. When these precipitates were obtained either by boiling five minutes or by lead acetate precipitation in the cold (5°C.), they contained no more than 10% of the total PSP available in the saline extracts. This small amount is regarded as insignificant and could be the result of simple physical adsorption on the precipitates and incomplete washing. The low toxicity of protein precipitates obtained under non-acidic conditions indicated that the pH effect in the bioassay may be involved in the low apparent toxicity of saline extracts when assayed without acidification. Studies on the effect of pH on the toxicity of purified PSP were undertaken in an effort to determine whether this effect could account for the results. Two solutions of purified PSP were made up in identically the same way, except that one (Solution A) was made up at pH 4.50 and the other (Solution B) at pH 7.25. These solutions were bioassayed periodically for sixteen days. As shown in the Table IX the toxicity of Solution B at pH 7.25 dropped to 78% of its original in one day and remained constant thereafter. Solution A retained its original toxicity during the entire time. On the sixteenth day half of Solution B was acidified to pH 3. The toxicity then increased to 93% of its original and remained constant at least an additional day.

These data indicate that the effect of pH on the toxicity of PSP can account for the low apparent toxicity observed in unacidified saline extracts of clam siphon tissue.

III. Projected Research for First Quarter, FY 1963

In view of the protection afforded mice against the lethal effects of paralytic shellfish poison by the sera from rabbits injected with PSP-HCHO-BSA, studies will be extended on the nature of the antibody responses elicited by these antigens. Techniques will also be investigated to reduce toxicity and to impart better antigenic properties to these conjugates.

IV. Isolation and Purification of Paralytic Shellfish Poison

To date 1010 pounds of toxic clam siphons have been received from Alaska. Assuming 70% yield in the purification process, these siphons represent the equivalent of about 6.8 grams of purified PSP.

A procedure has been developed for the isolation and purification of the poison which utilizes the best features of the methods described by Schantz et al (2) and those developed in connection with the quantitative chemical assay procedure of McFarren et al (3). Briefly, the process consists of

(a) preparation of a trichloroacetic acid extract of clam siphons,

(b) absorption and elution of the extract on IRC 50 ion exchange column at pH 5.2,

(c) absorption and elution of the first column eluate on the GC 50 ion exchange column at pH 5.2,

(d) conversion of second column eluate from an aqueous to an alcoholic solution,

(e) chromatographic purification of the alcoholic solution of PSP on an activated alumina column,

(f) concentration and drying of the purified alcohol extract.

One 100-lb lot of ground clam siphons has been partially purified by passing through the IRC 50 and GC 50 ion exchange columns. Some loss of poison was experienced due to the operation of the column at above optimum flow rates and as a result of a miscalculation related to the capacity of the IRC 50 resin. It is believed that these difficulties have now been overcome and the second batch of clam siphons is being put through the initial purification step. Also the partially purified PSP from the first lot is being carried through the remaining steps. Thus far there appear to be no unforeseen difficulties associated with this phase of the purification process.

V. References

1. Seventh Quarterly Report of Progress (January 1 - March 30, 1962) Research Project Number 4B04-14-004, Order Number FDO-3013.
2. Schantz, E.J., Mold, J.D., Stanger, D.W., Shavel, J., Riel, F.J., Bowden, J.P., Lynch, J.M., Wyler, R.S., Riegel, B., and Sommer, H. Paralytic Shellfish Poison. VI. A Procedure for the Isolation and Purification of the Poison from Toxic Clam and Mussel Tissues. J.A.C.S., 79:5230, 1957.
3. McFarren, E.F., Schantz, E.J., Campbell, J.E., and Lewis, K.H., Chemical Determination of Paralytic Shellfish Poison in Clams. J.A.O.A.C. 41:168, 1958.

Table I

Precipitin Titer of Rabbit Anti PSP-HCHO-BSA Sera after Immunization with 25 mg. of PSP-HCHO-BSA Antigen Protein

Serum No.*	Antigens 0.4 ml./tube	Dilutions of Antigens									
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
18	PSP-HCHO-BSA	-	-	-	±	+	+	+	+	+	+
	HCHO-BSA	-	-	-	+	+	+	+	+	+	+
19	PSP-HCHO-BSA	-	-	+	2+	4+	4+	3+	3+	2+	+
	HCHO-BSA	-	-	+	+	2+	4+	4+	3+	2+	+
21	PSP-HCHO-BSA	-	-	-	±	±	+	+	+	+	±
	HCHO-BSA	-	-	-	+	+	+	+	+	+	+

*Serum diluted 1:5, 0.4 ml. per tube.

Serum-saline controls negative.

Antigen-saline controls negative.

Table II

Precipitin Titer of Rabbit Anti PSP-HCHO-BSA Sera after Immunization with 50 mg. of PSP-HCHO-BSA Antigen Protein

Serum No.*	Antigens 0.4 ml./tube	Dilutions of Antigen									
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
18	PSP-HCHO-BSA	-	-	-	-	-	-	-	-	-	-
	HCHO-BSA	-	-	-	-	-	-	-	-	-	-
19	PSP-HCHO-BSA	3+	-	+	-	4+	4+	4+	4+	3+	+
	HCHO-BSA	3+	±	2+	4+	4+	4+	2+	+	±	-
21	PSP-HCHO-BSA	-	-	+	-	-	±	+	2+	-	-
	HCHO-BSA	-	±	+	2+	+	+	±	±	-	-

*Serum diluted 1:5, 0.4 ml. per tube.

Serum-saline controls negative.

Antigen-saline controls negative.

Table III

Passive Immunization of 19 to 21 g. White Mice to PSP by IP Injection of Rabbit Anti PSP-HCHO-BSA Sera Produced in Response to 25 mg. of Antigen Protein

Dilution of Serum Injected[a]	Mouse Number	Time of Death[b]
Rabbit 19 preimmunization Normal serum	1	4 min. 10 sec.
	2	7 min. 20 sec.
	3	6 min. 10 sec.
Rabbit 19 serum Undiluted	4	No death
	5	No death
	6	No death
Rabbit 19 serum Diluted 1:2	7	14 min. 44 sec.
	8	21 min. 31 sec.
	9	26 min. 24 sec.
Rabbit 19 serum Diluted 1:3	10	No death
	11	19 min. 3 sec.
	12	16 min. 33 sec.
Rabbit 19 serum Diluted 1:5	13	14 min. 52 sec.
	14	14 min. 49 sec.
	15	14 min. 19 sec.
Rabbit 19 serum[c] Undiluted	16	No death
	17	No death
	18	No death
Sterile distilled Water	16	6 min. 0 sec.
	17	5 min. 43 sec.
	18	6 min. 12 sec.

(a) Mice received two 1.0 ml. IP injections of serum; 1.0 ml. 24/hours prior to challenge followed by a second 1.0 ml. dose 4 hours prior to challenge.

(b) Mice were challenged by IP injection of 1.0 ml. of aqueous solution of PSP containing approximately 0.289 micrograms per ml.

(c) Mice received one 1.0 ml. IP injection of serum 4 hours prior to challenge.

Table IV

Passive Immunization of 19 to 21 g. White Mice to PSP by Injection of Rabbit Anti PSP-HCHO-BSA Sera Produced in Response to 50 mg. of Antigen Protein

Dilution of Serum Injected[a]	Mouse Number	Time of Death[b]
Rabbit 19 serum undiluted	1 2 3	No death No death No death
Rabbit 19 serum diluted 1:2	4 5 6	24 min. 7 sec. 19 min. 48 sec. 20 min. 0 sec.
Rabbit 19 serum diluted 1:3	7 8 9	8 min. 11 sec. 12 min. 22 sec. 17 min. 10 sec.
Rabbit 19 serum diluted 1:5	10 11 12	6 min. 17 sec. 7 min. 32 sec. 7 min. 40 sec.
Rabbit 19 serum undiluted[c]	13 14 15	No death No death No death
Sterile distilled water	16 17 18	5 min. 29 sec. 6 min. 55 sec. 4 min. 53 sec.

(a) Mice received two 1.0 ml. IP injections of serum; 1.0 ml. 24 hours prior to challenge followed by a second 1.0 ml. dose 4 hours prior to challenge.

(b) Mice were challenged by IP injection of 1.0 ml. of aqueous solution of PSP containing approximately 0.299 micrograms per ml.

(c) Mice received one 1.0 ml. IP injection of serum 4 hours prior to challenge.

Table V

Passive Immunization of 19 to 21 g. White Mice to PSP by Injection of Rabbit Anti PSP-HCHO-BSA and HCHO-BSA Sera Produced in Response to 50 mg. of Antigen Protein

Type of Serum Injected Undiluted[a]	Mouse Number	Time of Death[b]
Rabbit 18 Anti PSP-HCHO-BSA serum	1	8 min. 29 sec.
	2	8 min. 6 sec.
	3	7 min. 36 sec.
Rabbit 21 anti PSP-HCHO-BSA serum	4	9 min. 27 sec.
	5	9 min. 29 sec.
	6	12 min. 24 sec.
Rabbit 6 anti HCHO-BSA serum	7	6 min. 56 sec.
	8	8 min. 31 sec.
	9	5 min. 40 sec.
Sterile distilled water	10	5 min. 29 sec.
	11	6 min. 55 sec.
	12	4 min. 53 sec.

(a) Mice received two 1.0 ml. IP injections of serum; 1.0 ml. 24 hours prior to challenge followed by an additional 1.0 ml. dose 4 hours prior to challenge.

(b) Mice were challenged by IP injection of 1.0 ml. of aqueous solution of PSP containing approximately 0.299 micrograms per ml.

Table VI

Precipitin Titer of Rabbit Sera Produced in Response to I.P. Injections of Gonyaulax catenella cellular Homogenate Diluted 1:20 in Water

Serum number*	Antigens 0.4 ml./tube	Dilutions of Antigens									
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
12 - after receiving 60 micrograms PSP equivalent.	G.catenella	+	+	+	+	±	-	-	-	-	-
	PSP-HCHO-BSA	-	-	-	-	-	-	-	-	-	-
12 - after receiving 120 micrograms PSP equivalent.	G.catenella	+	+	+	2+	2+	+	±	±	-	-
	PSP-HCHO-BSA	-	-	-	-	-	-	-	-	-	-
12 - after receiving 180 micrograms PSP equivalent.	G.catenella	+	+	+	2+	2+	2+	+	±	-	-
	PSP-HCHO-BSA	-	-	-	-	-	-	-	-	-	-
12 - after receiving 240 micrograms PSP equivalent.	G.catenella	+	+	+	2+	2+	2+	+	+	-	-
	PSP-HCHO-BSA	-	-	-	-	-	-	-	-	-	-
16 - after receiving 60 micrograms PSP equivalent.	G.catenella	+	±	-	-	-	-	-	-	-	-
	PSP-HCHO-BSA	-	-	-	-	-	-	-	-	-	-
20 - after receiving 45 micrograms PSP equivalent.	G.catenella	-	-	-	±	+	+	±	-	-	-
	PSP-HCHO-BSA	-	-	-	-	-	-	-	-	-	-

*Serum diluted 1:5, 0.4 ml. per tube.

Serum saline and antigen saline controls negative.

Table VII

Passive Immunization of 19 to 21 gram White Mice to PSP by Injection of Rabbit Anti *Gonyaulax catenella* Sera

Serum injected undiluted[(a)]	Mouse Number	Time of Death[(b)]
Rabbit 12 antiserum produced in response to 240 micrograms PSP equivalent.	1 2 3	7 min. 15 sec. 5 min. 2 sec. 6 min. 36 sec.
Rabbit 16 antiserum produced in response to 60 micrograms PSP equivalent.	4 5 6	4 min. 55 sec. 5 min. 33 sec. 6 min. 0 sec.
Rabbit 20 antiserum produced in response to 45 micrograms PSP equivalent.	7 8 9	6 min. 58 sec. 5 min. 30 sec. 6 min. 15 sec.
Sterile distilled water	10 11 12	5 min. 29 sec. 6 min. 55 sec. 4 min. 53 sec.

(a) Mice received two 1.0 ml. I.P. injections of serum; 1.0 ml. 24 hours prior to challenge followed by an additional 1.0 ml. dose 4 hours prior to challenge.

(b) Mice were challenged by I.P. injection of 1.0 ml. of aqueous solution of PSP containing approximately 0.299 micrograms per ml.

Table VIII

Influence of Temperature and Acid Hydrolysis of Saline Extracts of Toxic Clam Siphons

Weight Siphon Tissue	Temperature of Extraction	Toxicity of Unacidified Extract	Toxicity of Acid Hydrolyzed Extract	% of Potential Toxicity in Unacidified Extract
2.10 g.	25-30° C.	1.31 µg PSP/ml	1.77 µg PSP/ml	73
2.68 g.	0-5° C.	1.72 µg PSP/ml	2.32 µg PSP/ml	74
3.14 g.	0-5° C.	2.09 µg PSP/ml	2.66 µg PSP/ml	78

Table IX

Influence of pH and Time on the Concentration of PSP Calculated from Mouse Bioassay

Time in Days	Solution A	Solution B	Solution C
	PSP at pH 4.50	PSP at pH 7.25	Solution B Acidified
0	13.2 µg/ml	13.3 µg/ml	-
1	13.2 µg/ml	10.4 µg/ml	-
8	13.1 µg/ml	11.5 µg/ml	-
10	13.4 µg/ml	10.3 µg/ml	-
17	-	10.6 µg/ml	12.1 µg/ml
18	-	-	[illegible] µg/ml